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METHODS AND COMPOSITIONS FOR DESTABILIZING MICROTUBULES

FIELD OF THE INVENTION

[0001] The following invention relates to polypeptides that possess microtubule-destabilizing activity, polynucleotides encoding such polypeptides, and compositions and methods of use thereof.

REFERENCES

[0002] The following publications are cited in this application:

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[0003] All of the publications cited above or elsewhere in this application are herein incorporated by reference in their entirety to the same extent as if the disclosure of each individual publication was specifically and individually indicated to be incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0004] Microtubules are essential for a variety of cellular functions, including maintenance of cell shape, cell movement, cell polarity, intracellular transport, mitosis, and meiosis. Microtubule networks exist in dynamic equilibrium and undergo dramatic reorganization through the course of the cell cycle. For example, upon entering mitosis, the interphase microtubule network is rapidly disassembled, followed by the reorganization of microtubules into the mitotic spindle.

[0005] Microtubules are comprised of α-β tubulin heterodimers, which are commonly assembled and nucleated by the γ-tubulin protein complexes resided in the centrosomes (Zheng et al., 1995; Erickson and Stoffler, 1996). Regulation of microtubule formation is controlled by two groups of proteins, namely microtubule stabilizers and destabilizers (Heald and Nogales, 2002). The former group is exemplified by microtubule-associated proteins (MAPs), which bind to and stabilize microtubules (Cassimeris and Spittle, 2001). The latter group includes Op18/stathmin (Belmont and Mitchison, 1996; Cassimeris, 2002), katanin (McNally and Vale, 1993; Hartman *et al.*, 1998), and Kin I kinesins (Walczak *et al.*, 1996; Desai *et al.*, 1999), which possess the ability to disassemble microtubules.

[0006] Protein 4.1R, originally identified as an 80-kDa protein (*i.e.*, 4.1R-80) in human erythrocytes, plays a crucial role in maintaining the specialized mechanical properties of erythrocyte plasma membranes. Multiple protein 4.1R isoforms, generated by alternative RNA splicing, have been identified in erythroid (Tang *et al.*, 1988; Conboy *et al.*, 1988) and nonerythroid (Tang *et al.*, 1990; Conboy *et al.*, 1991; Huang *et al.*, 1993) cells.

[0007] In addition to being associated with the plasma membrane cytoskeleton, protein 4.1R also localizes to intracellular sites in nucleated cells, including the nuclear matrix (De Career *et al.*, 1995; Krauss *et al.*, 1997a), microtubules (Perez-Ferreiro *et al.*, 2001), and centrosomes (Krauss *et al.*, 1997b; Hung *et al.*, 2000). A nonerythroid isoform of 4.1R (4.1R-135) has also been reported to interact with the nuclear mitotic apparatus protein (NuMA) in the interphase nucleus, forming a complex with several spindle-pole organizing proteins, including NuMA, dynein, and dynactin, during cell division (Mattagajasingh et al., 1999). The 4.1R-135 isoform is generated by alternative mRNA splicing, which produces a mRNA encoding a polypeptide having an additional 209 N-terminal amino acid residues, relative to the erythroid 4.1R-80 isoform (Tang *et al.*, 1990). The subcellular localization and the interaction of the nonerythroid 4.1R-135 isoform with NuMA suggest an essential role of 4.1R-135 in organizing the architecture of the nucleus and mitotic spindle (Mattagajasingh et al., 1999).

[0008] Using the N-terminal head region of 4.1R-135 (*i.e.*, amino acid residues 1-209) in a yeast two-hybrid screening assay, a centrosomal protein designated centrosomal P4.1-associated protein (CPAP) was identified (Hung *et al.*, 2000). In addition to binding to the head region of 4.1R-135, CPAP is associated with the γ-tubulin complex. Although increasing numbers of factors have been identified that are important for microtubule functions, their mode of action and practical use are yet to be elucidated.

SUMMARY OF THE INVENTION

[0009] The invention provides polypeptides, and polynucleotides encoding such polypeptides, that are useful for destabilizing microtubules. Since microtubules play an essential role in cell division, which occurs more frequently in tumor cells, the polypeptides and polynucleotides of the present invention can be used to treat tumors.

[0010] Accordingly, one aspect of the invention provides an isolated polypeptide selected from the group consisting of:

a) a polypeptide of amino acid residues 311-422 of SEQ ID NO:1 (Figure 6; GenBank AAG21074; Hung et al., 2000); and
b) a polypeptide that shares substantial sequence similarity with residues 311-422 of SEQ ID NO:1 and has microtubule destabilizing activity.

[0011] In one embodiment of the invention, the polypeptide is a recombinant polypeptide, for example, a fusion polypeptide comprising amino acid residues 311-422 of SEQ ID NO:1, or a polypeptide that shares substantial sequence similarity thereto and has microtubule destabilizing activity. The recombinant polypeptide may comprise additional flanking or internal polypeptide sequences that modulate expression, solubility, activity, specificity, localization, or stability, or that facilitate purification or identification.

[0012] The polypeptide may be synthetic, and comprise artificial or modified amino acid residues. The synthetic polypeptide may also comprise additional amino acid residues that modulate solubility, activity, specificity, localization, or stability, or that facilitate synthesis, purification or identification.

[0013] In another embodiment, the invention provides a polynucleotide encoding the polypeptide described above. In one particular embodiment of the invention, the polynucleotide comprises nucleotide 1141 to nucleotide 1476 of SEQ ID NO:2 (Figure 7; GenBank AF139625; Hung et al., 2000).

[0014] In one embodiment, the invention provides a method for destabilizing microtubules in a cell, comprising providing to the cell a polypeptide in a sufficient amount to destabilize microtubules in the cell, wherein the polypeptide is selected from the group consisting of:

a) a polypeptide of amino acid residues 311-422 of SEQ ID NO:1;
b) a polypeptide that shares substantial sequence similarity with residues 311-422 of SEQ ID NO:1 and has microtubule destabilizing activity,
c) a polypeptide of SEQ ID NO:1; and
d) a polypeptide that shares substantial sequence similarity with SEQ ID NO:1 and has microtubule destabilizing activity.

[0015] In a further embodiment, the invention provides a method for inhibiting proliferation of a cell, comprising providing to the cell a polypeptide of the present invention in a sufficient amount to inhibit proliferation of the cell.

[0016] In yet another embodiment of the invention, the invention provides a method for treating a tumor, comprising providing to cells of the tumor a polypeptide of the present invention in a sufficient amount to inhibit proliferation of the cell.

[0017] The polypeptide can be provided as a polynucleotide that encodes the polypeptide, such as an expression vector. In one embodiment of the invention, the polypeptide is expressed using an inducible promoter.

[0018] In one embodiment of the invention, the expression vector specifically or preferentially replicates in the cells in which microtubule destabilization is desired, particularly tumor cells. The vector is preferably derived from a virus, for example, a virus selected from the group consisting of retroviruses, adenoviruses, adeno-associated viruses, pox viruses, or herpesviruses. In a particular embodiment of the invention, the vector is derived from a lentivirus.

[0019] In still another embodiment, the invention provides a method for screening drugs that modulate microtubule assembly, comprising contacting microtubules with a polypeptide of the present invention, in the presence and absence of a drug, and determining the effect of the drug on microtubule assembly. The polypeptide may be any PN2-3 polypeptide, or a compound comprising a PN2-3 polypeptide. In a particular embodiment of the invention, such drugs modulate microtubule assembly by modulating PN2-3.

[0020] In yet another embodiment of the invention, the above-described polypeptides, or polynucleotides encoding such polypeptides, are provided to cells in the presence of one or more drugs that affect microtubule assembly or stability. For example, the polypeptides of the invention can be used to destabilize taxol-stabilized microtubules, such as would be found in a mammal receiving taxol to reduce the risk of restenosis following stent implantation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] **Figure 1** shows inhibition of microtubule nucleation from purified centrosomes by polypeptides derived from CPAP. (A) Diagram depicting cDNA constructs encoding various portions of CPAP. (B) Coomassie-stained polyacrylamide gel showing affinity-purified GST-truncated CPAP fusion proteins. (C) Results of an *in vitro* microtubule nucleation assay showing the effect of truncated CPAP fusion proteins on microtubule nucleation. (D) Quantitative analysis of the inhibitory effects observed in (C).

[0022] **Figure 2** shows inhibition of microtubule nucleation by portions of the PN2 domain of CPAP. (A) Diagram depicting cDNA constructs encoding various portions of the PN2 domain of CPAP. (B) Coomassie-stained polyacrylamide gel showing affinity-purified GST-truncated PN2 CPAP fusion proteins. (C and D) Results of an *in vitro* microtubule nucleation assay showing the effect of truncated PN2 CPAP fusion proteins on microtubule nucleation. (E) Results of an *in vitro* microtubule destabilization assay performed in the absence of centrosomes and in the presence of truncated PN2 CPAP fusion proteins.

[0023] **Figure 3** shows inhibition of microtubule nucleation by portions of the PN2-3 fragment of CPAP. (A) Diagram depicting cDNA constructs and synthetic peptides encoding various portions of the PN2-3 fragment of CPAP. (B) Coomassie-stained polyacrylamide gel showing affinity-purified GST-truncated PN2-3 CPAP fusion proteins. (C and D) Results of an *in vitro* microtubule nucleation assay showing the effect of truncated PN2-3 CPAP fusion proteins (C) and the synthetic peptide derived from PN2-3 (D) on microtubule nucleation. (E) Quantitative analysis of the inhibitory effects observed in (C). (F) Location of the proline substitutions in four mutated PN2-3 polypeptides and the predicted relative probability (compared to the wild-type sequence (*i.e.*, SEQ ID NO:1)) that each mutated polypeptide will form an α-helical domain. (G) Results of an *in vitro* microtubule nucleation assay using the polypeptides of (F) along with wild-type PN2-3.

[0024] **Figure 4** shows the results of microtubules sedimentation assays. (A) Purified tubulins were incubated with various amounts (as indicated) of GST-PN2-3 (top panel) or GST-PN2-2 (bottom panel). Control samples received no CPAP-derived polypeptides (first two lanes of each panel). The supernatants (S) and pellets (P) were analyzed by SDS-PAGE following electrophoresis. (B) Similar to (A) with the addition of taxol to stabilize the microtubules.

[0025] **Figure 5** shows the results of flow cytometry analysis of cells transfected with GFP or GFP-PN2-3 expression vectors. The horizontal and vertical axes represent relative DNA content and cell number, respectively.

[0026] **Figure 6** shows the amino acid sequence of CPAP (SEQ ID NO:1). Amino acids 311-422 are underlined.

[0027] **Figure 7** shows the cDNA sequence that codes for CPAP (SEQ ID NO:2). Nucleotides 1141-1476 are underlined.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Prior to describing the invention in further detail, the terms used in this description are defined as follows unless otherwise indicated.

Definitions

[0029] *Administering (to cells)*: Delivering the object of the invention to a target population of cells. As used herein, *administering* encompasses any methodology, route of delivery, and technique useful for bringing the polypeptides and/or polynucleotides of the invention into contact with the target population of cells, including but not limited to, infection, transfection, transduction, ballistic delivery, microinjection, and any form of receptor or carrier-mediated transport.

[0030] *Depolymerizing (depolymerization of) microtubules*: Depolymerizing (depolymerization of) microtubules: The process of disassembling microtubules into their component subunits (usually α-β heterodimers).

[0031] *Domain*: As used herein, domains of polypeptides are regions that possess a characteristic structure or function. Examples of domains include α-helices, β-sheets, β-bends, loops, and unstructured polypeptide regions between structured regions.

[0032] *Expressing a polypeptide in the target cells*: Causing target cells to produce, via protein translation, a polypeptide sequence of interest (*e.g.*, CPAP or a portion thereof) from a polynucleotide encoding such polypeptide.

[0033] *Expression vector*: A vector that encodes one or more polypeptides of interest along with appropriate transcriptional and translational regulatory sequences to allow expression of the polypeptide in a cell. Examples of expression vectors include plasmids, phages, and viruses. Expression vectors may comprise inducible or cell-type-specific promoters, enhancers or repressors, introns, polyadenylation signals, selectable markers, polylinkers, site-specific recombination sequences, and other features to improve functionality, convenience of use, and control over mRNA and/or protein expression levels, as known in the art.

[0034] *Fusion protein*: A contiguous polypeptide comprising polypeptide sequences derived from a plurality of sources.

[0035] *Inducible promoter*: A promoter that causes RNA to be transcribed from a particular polynucleotide sequence at different levels depending upon specific intracellular or environmental conditions. Inducible promoters may respond positively or negatively to, for example, the presence of hormones, nutrients, metabolites, toxins, stress, osmolarity, the activation or inactivation of certain cellular biochemical pathways, or other means of regulating gene expression.

[0036] *Inhibiting proliferation of a cell (inhibits proliferation)*: Decreasing the number of mitotic divisions of a cell within a given amount of time. Inhibition may be partial, such that the cell continues to undergo mitotic division but at a decreased rate compared to a similar cell in a comparable environment, or complete, such that the cell ceases to undergo mitotic division.

[0037] *Microtubule destabilizing activity*: The activity of preventing, inhibiting, or reversing the polymerization of microtubules. Thus, a microtubule destabilizing activity may be the activity to inhibit microtubule nucleation or depolymerize existing microtubule either *in vitro* or *in vivo*.

[0038] *Modulate microtubule assembly*: To cause disassembly or assembly of microtubules or to shift the dynamic equilibrium of microtubule formation so as to favor the formation of free tubulin or microtubule.

[0039] *Naturally occurring variant*: A polynucleotide or polypeptide sequence that is found in nature and that possesses a high degree of structural and functional identity to a subject polynucleotide or polypeptide. Naturally occurring variants include but are not limited to polynucleotides or polypeptides having different transcriptional or translation start and/or stop positions, respectively; splice variants; variants resulting from gene rearrangement, duplication, inversion, deletion, and/or recombination; allelic variants; variants resulting from RNA editing; and other events know to alter polynucleotide and/or polypeptide sequences. Preferably, such variants have at least 98%, at least 95%, or at least 90% polypeptide sequence identity with respect to a particular polynucleotide or polypeptide sequence of interest.

[0040] *PN2-3 activity*: A biological activity of PN2-3 disclosed herein, such as the microtubule destabilizing activity, proliferation-inhibiting activity, or cell cycle arrest activity. Microtubule destabilizing activity can be determined by any method known in the art, preferably one of the assays described or referenced herein.

[0041] *PN2-3 polypeptide*: A polypeptide having substantial sequence similarity with residues 311-422 of CPAP (SEQ ID NO:1) and having at least one PN2-3 activity. The polypeptide preferably has a helical region and a non-helical region as predicted by any software available in the art. A PN2-3 polypeptide in which the helical and non-helical regions are switched, yet retaining at least one PN2-3 activity, is also contemplated herein.

[0042] *PN2-3 sequence* or *PN2-3 region*: Amino acid sequence of residues 311-422 of CPAP (SEQ ID NO:1).

[0043] *Polypeptide*: A molecule having any number of contiguous amino acid residues linked via peptide bonds. As used herein, *polypeptide*, *peptide*, and *protein* are interchangeable.

[0044] *Providing to the cell*: Bringing into contact with a cell or causing to be taken up by a cell.

[0045] *Substantial sequence similarity*: With respect to polypeptides, substantial sequence similarity exists when two polypeptide sequences are at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 95% identical at the primary sequence level, as determined by a sequence alignment program available in the art, such as Clustal W.

[0046] *Sufficient amount*: An amount that is effective in achieving an intended result or obtaining a desired effect.

[0047] *Taxol-stabilized microtubules*: Microtubules that are resistant to depolymerization due to the presence of taxol.

[0048] *Treating or ameliorating a disease or medical condition*: reducing or completely removing the symptoms of a disease or medical condition.

[0049] *Tumor cells, also known as neoplastic cells:* Cells which proliferate without the normal growth inhibition properties. A new growth comprising neoplastic cells is a *neoplasm* or *tumor*. A neoplasm is an abnormal tissue growth, generally forming a distinct mass, that grows by cellular proliferation more rapidly than normal tissue growth. Neoplasms may show partial or total lack of structural organization and functional coordination with normal tissue. As used herein, a neoplasm is intended to encompass hematopoietic neoplasms as well as solid neoplasms.

[0050] A neoplasm may be benign (benign tumor) or malignant (malignant tumor or cancer). Malignant tumors can be broadly classified into three major types. Malignant neoplasms arising from epithelial structures are called carcinomas, malignant neoplasms that originate from connective tissues such as muscle, cartilage, fat or bone are called sarcomas and malignant tumors affecting hematopoietic structures (structures pertaining to the formation of blood cells) including components of the immune system, are called leukemias and lymphomas. Other neoplasms include, but are not limited to neurofibromatosis.

[0051] A disease or medical condition of which the symptoms include a tumor may also be referred to as a *tumor* or *neoplasm*.

Detailed Description of the Invention

[0052] The invention provides a 112-amino acid residue polypeptide derived from centrosomal P4.1-associated protein (CPAP; SEQ ID NO:1) that possesses the ability to inhibit microtubule formation. The polypeptide contains residues 311-422 of CPAP. The invention further provides polynucleotides encoding such polypeptides, and compositions and methods of use thereof.

[0053] The polypeptide was discovered as a result of a structure-function analysis of CPAP. Briefly, an *in vitro* microtubule nucleation assay was developed to determine whether the CPAP polypeptide comprised a functional portion that affected microtubule assembly nucleated from a centrosome. As described in Example 1, polynucleotide sequences encoding truncated forms of the CPAP polypeptide were cloned into a glutathione-S-transferase (GST)-expression vector, PGEX-2T, for expression of GST-truncated CPAP polypeptides. These GST-truncated CPAP proteins were then tested for their ability to affect microtubule formation. The results indicate that PN2, residues 132-422 of CPAP, inhibited microtubule formation.

[0054] To more precisely define the region of PN2 that was responsible for inhibiting microtubule nucleation, expression vectors were constructed to express GST-fusion polypeptides comprising one of three sub-regions of PN2. Only GST-PN2-3 (corresponding to residues 311-422 of CPAP) possessed the ability to inhibit microtubule assembly, indicating that the portion of PN2 necessary to inhibit centrosome-dependent microtubule nucleation resides in the PN2-3 region.

[0055] The CPAP polypeptide comprises five α-helical coiled-coil motifs, designated I to V, which are punctuated by a helix-disrupting proline residue (Hung *et al.*, 2000; Figure 3A). The PN2-3 region of CPAP comprises both an α-helical domain (residues 327-362) and a non-helical structure (residues 363-422). To examine the role of this α-helical structure, the PN2-3 polypeptide was further divided into three sub-regions that comprised the α-helical motif (PN2-3N), the non-helical motif (PN2-3C), or both (PN2-3M). The polypeptides inhibited microtubule nucleation with the following relative level of efficacy: GST-PN2-3 > GST-PN2-3M > GST-PN2-3C. No inhibition was observed in the case of GST-PN2-3N, which comprises only the α-helical motif. Accordingly, both the α-helical and the non-helical structures in PN2-3 are required for the most effective inhibition of microtubule nucleation from the centrosomes.

[0056] To further define the amino acid residues and/or polypeptide secondary structures responsible PN2-3 activity, four residues within the putative amino acid sequence of α-helix domain II (residues 327-362) were mutated to the known helix-disrupting residue, proline. Thus, Y341, I346, L348, and L353 were individually mutated to proline. In the microtubule nucleation assay, only the wild-type and L353P polypeptides inhibited microtubule assembly. Therefore, the amino acid residues at positions 341, 346, and 348 (but not 353) are important for PN2-3 activity, presumably due to their structural role in forming or maintaining α-helical domain II.

[0057] By expressing PN2-3 or its related polypeptides in cultured cells, it was further discovered that the polypeptides inhibit cell proliferation and cause G_2/M arrest in the cell cycle. Therefore, the polypeptides can be used to inhibit microtubule formation and cell proliferation.

[0058] Accordingly, the present invention provides a polypeptide that is derived from CPAP and possesses the ability to sequester or disassemble microtubules. The most active polypeptide (PN2-3) was a 112-amino acid residues in length and comprised both a predicted α-helical structure (residues 327-362) and non-helical structure (residues 363-422) of CPAP. No significant sequence homology was found between PN2-3 and other known microtubule destabilizing polypeptides, including OP18/stathmin (Belmont and Mitchison, 1996; Cassimeris, 2002), katanin (McNally and Vale, 1993; Hartman *et al.*, 1998), and Kin I kinesins (Walczak *et al.*, 1996; Desai *et al.*, 1999). Therefore, the PN2-3 polypeptide represents a novel type of microtubule destabilizing sequence.

[0059] Without being limited to a theory, it is believed that the PN2-3 polypeptide directly recognizes the "plus ends" of microtubules, which results in the inhibition of microtubule nucleation from centrosomes. This mechanism is supported by the observation that GST-PN2-3 and GST-CM localize to the plus ends of newly assembled microtubules, formed in the presence of centrosomes (data not shown). However, in the absence of centrosomes, the PN2-3 polypeptide also destabilizes microtubules, suggesting that the PN2-3 polypeptide may bind to tubulin subunits, causing the disassembly of existing microtubules. The ability of the PN2-3 polypeptide to both inhibit microtubule nucleation from centrosomes and cause the disassembly of microtubules make PN2-3 particularly effective as a substance for modulating microtubule assembly in cells, including but not limited to rapidly proliferating cells, such as cancer cells.

[0060] Accordingly, in one embodiment, the instant invention provides the 112-amino acid residue-PN2-3 polypeptide, which comprises amino acid residues 311-422 of

CPAP (SEQ ID NO:1). The PN2-3 polypeptide possesses the ability to inhibit microtubule formation in various assays, including inhibiting the formation of microtubule nucleated from centrosomes and destabilize polymerized tubulin. Also provided are PN2-3 polypeptides that have substantial sequence similarity with amino acid residues 311-422 of CPAP, as well as a PN2-3 activity. PN2-3 activity can be determined, for example, by using any of the biological assays described herein. A PN2-3 polypeptide has preferably about 50-500 amino acids, more preferably about 75-400 amino acids, yet more preferably about 100-300 amino acids, still more preferably about 100-250 amino acids, and most preferably about 100-200 amino acids.

[0061] Such polypeptides may be derived from naturally-occurring variants of CPAP or may be engineered polypeptides. In a preferred embodiment of the invention, such polypeptides comprise conservative amino acid substitutions, which are well known to those skilled in the art. Conservative amino acid substitutions include but are not limited to the substitution of amino acids with residues of similar charges, sizes, and/or hydrophobic and hydrophilic characteristics.

[0062] One embodiment of the invention provides a polypeptide comprising the PN2-3 sequences in the context of a larger polypeptide, including but not limited to the full-length CPAP polypeptide or portions thereof, provided that the resulting polypeptides demonstrate PN2-3 activity. A particular embodiment of the invention provides the CM polypeptide (comprising amino acid residues 132-607 of CPAP). Another embodiment of the invention provides the PN2 polypeptide (comprising amino acid residues 132-422 of CPAP).

[0063] Yet another embodiment of the invention includes a portion of the PN2-3 polypeptide, provided that the portion of the PN2-3 polypeptide demonstrates PN2-3 activity. In one embodiment of the invention, the portion of PN2-3 is PN2-3M, which comprises amino acid residues 320-390 of CPAP. Other portions of PN2-3 may also retain PN2-3 activity. For example, PN2-3C, which comprises amino acid residues 369-422 of CPAP, is known to retain some PN2-3 activity, although it is less active than PN2-3M. Additional polypeptides of the invention may be identified by expressing or synthesizing different portions of the PN2-3 region and assaying the polypeptides for PN-2-3 activity, using any of the methods described herein. Other methods available in the art for determining microtubule destabilizing activities can also be employed.

[0064] The invention also provides a fusion polypeptide comprising a PN2-3 polypeptide along with additional amino acid sequences. These additional sequences may facilitate expression, purification, identification, solubility, membrane transport, stability, activity, localization, toxicity, or specificity of the resulting polypeptide.

[0065] In one embodiment of the invention, the additional amino acid sequences encode a portion of glutathione-*S*-transferase (GST), which allows affinity purification of the fusion polypeptide. In a particular embodiment of the invention, a protease cleavage site is provided between the GST sequence and CPAP sequence that comprises the PN2-3 sequences, such that the GST sequences may be removed by a protease that recognizes the cleavage site. Proteases and cognate recognition sequences that are useful for practicing the invention include, but are not limited to, thrombin or Factor VIII. Virtually any affinity tag may be expressed as part of the fusion polypeptide, provided that it does not cause irreversible misfolding and degredation of the fusion polypeptide. Similarly, virtually any protease is useful for cleaving an affinity tag when a cognate recognition sequence is provided between the tag and the PN2-3 sequence, so long as the protease does not affect PN2-3 activity, for example, by cleaving the PN2-3 polypeptide internally.

[0066] In a related embodiment of the invention, the additional amino acid sequences may provide a mechanism for modulating the activity of the expressed polypeptide such that the PN2-3 activity is only present in particular types of cells (*i.e.*, target cells). In one embodiment, the additional sequences may resemble the plus ends of microtubules or tubulin subunits, such that the PN2-3 polypeptide of the fusion protein binds to the "decoy" sequence, thereby preventing or inhibiting the PN2-3 polypeptide from interacting with cellular microtubules. In a most preferred embodiment of the invention, such a fusion protein would further comprise a protease cleavage site between the PN2-3 polypeptide and the decoy portion of the fusion protein. The decoy portion could then be removed by a protease present specifically, or predominantly, in the target cells.

[0067] In a preferred embodiment of the invention, such a protease could be endogenous to the target cells. For example, the protease cathepsin D is known to be over-expressed in breast cancer cells, and is believed to be responsible for increased invasiveness (Rochefort, 1990). Urokinase-type plasminogen activator (uPA), cathepsin B, and matrix metalloproteinases MMP2 and MMP9 are known to be present at increased levels in high-grade astrocytomas, relative to levels in normal cells or low-grade astrocytomas, and are believed to degrade the extracellular matrix (ECM) (Rao, 2003). A protease known to be overexpressed by certain cancer cells, or known to be over-expressed at sites of metastatic invasion, could be used to activate (*i.e.*, cleave the region responsible for modulating the activity of) the PN2-3 polypeptide, specifically or preferentially in the target cells. Such an arrangement provides precise control over PN2-3 activity and allow the selective killing of target cells, particularly cancer cells.

[0068] In an alternative embodiment of the invention, an additional expression vector is provided to the mixed population of cells, wherein the additional expression vector encodes a protease under the control of a promoter that is specifically or preferentially active in the target cells. Such a promoter could be selected by identifying proteins that are overexpressed in a target cell type and using a promoter from a gene encoding such proteins to control expression of the protease. Accordingly, a polypeptide having PN2-3 activity would only be generated in cells that expressed the protease.

[0069] The additional sequences may also be used to target delivery of the fusion polypeptide. For example, the additional sequences may correspond to a polypeptide ligand specific for a receptor or surface molecule that is differentially-expressed, presented, or modulated in target cells. Any such additional sequences may be used, provided that the presence of the PN2-3 region does not abolish the ability of the cell-type specific portion of the fusion polypeptide to be recognized and internalized by a cell possessing the appropriate cell surface molecules and that the additional sequences do not interfere with PN2-3 activity. Optionally, the cell-type specific portion of the polypeptide may be cleaved by a protease, as described above.

[0070] Polypeptides comprising other amino acid substitutions, deletions, or insertions, with respect to the PN2-3 region, may also be useful for practicing the invention provided that they retain PN2-3 activity and/or specificity. As discussed above, substitutions that disrupt α-helical domain II (*e.g.*, Y341P, I346P, and L348P) appear to reduce or abolish PN2-3 activity, while the L353 substitution does not affect the activity. Polypeptides comprising non-naturally-occurring amino acids, non-amino acid moieties (including but not limited to, carbohydrate and lipid moieties) may also be useful for practicing the invention. Such polypeptides may be synthetic polypeptides and may include any number of non-naturally-occurring amino acids and/or additional functional groups (including but not limited to fluorescence and/or quenching groups), provided that the resulting polypeptides retain PN2-3 activity.

[0071] The invention also includes methods of using the polypeptides of the invention. In one embodiment, a PN2-3 polypeptide is administered to eukaryotic cells to destabilize microtubules in the cells, or prevent the assembly of microtubules. In a preferred embodiment of the invention, administration of the polypeptide decreases the rate of proliferation of such cells. In a particular embodiment of the invention, administration of the polypeptide prevents the cells from proliferating by causing G_2/M arrest. In a most preferred embodiment of the invention, the cells are killed.

[0072] Numerous methods of introducing polypeptides into cells are known in the art, including but not limited to transfection, microinjection, scrape-loading, and receptor-mediated uptake by the cell. Transfection may be transient or stable. Exemplary current methods of transfection include, calcium phosphate precipitation, electroporation, lipofection, and peptide-mediated transfection. Ballistic DNA delivery and transduction (*i.e.*, the introduction of foreign DNA by virus or virus vector infection) can also be employed.

[0073] For example, polypeptides of the present invention can be delivered to cells by means of an expression vector. Suitable expression vectors comprise a promoter that is active in the cells in which a PN2-3 polypeptide is to be expressed (*i.e.* target cells). For example, in the case of cancer cells, the promoter may be derived from a growth factor that is known to be overexpressed in the particular cancer cell. Alternatively, the promoter may be active during a certain stage of the cell cycle, such as the G_2/M transition. In this manner, quiescent cells will not express the polypeptide comprising PN2-3 sequences, while cells entering the G_2/M transition in the cell cycle will express such polypeptides. This feature of this particular embodiment of the invention may be desirable to prevent the expression of PN2-3 in nerve cells, which rely on extensive processes (involving elaborate microtubule architecture) for cell-cell communication. Since nerve cells are slow growing, and in some cases essentially quiescent (*e.g.*, in the brain), promoters that are specific for the M or S phases of the cell cycle are unlikely to function in nerve cells.

[0074] Expression vectors useful for practicing the invention may also include selectable markers, cell-type or cell-cycle-specific enhancers or repressors, polylinkers, start codons, ribosome binding sites, internal ribosome entry sites, introns, stop codons, polyadenylation signals, or other features that facilitate cloning and vector stability, mRNA stability and localization in the cell, and translation efficiency, or combinations thereof. Expression vectors include viral expression vectors. Selection of these features is largely based on the target cells to which PN2-3 is to be delivered and the expression characteristics desired. A large number of commercially available vectors are available for expressing polypeptides in cells.

[0075] In one embodiment of the invention, the polynucleotides encoding the PN2-3 polypeptide are delivered to cells using a viral vector. Numerous viruses are known to be useful for delivering polynucleotides to animal cells (reviewed in Todd *et al.*, 2000), including but not limited to adenoviruses, adeno-associated viruses, retroviruses (including lentiviruses), herpesviruses, and pox viruses. Plant viruses useful for delivering the polypeptides of the invention to plants include but are not limited to tobacco mosaic virus, cauliflower mosaic virus, alfalfa mosaic virus, and potato virus X. Fungal and protozoan species are also known to be susceptible to viral infection (Fields 1996 at pages 557-585; McCabe *et al.*, 1999), providing a means to deliver polynucleotide sequences that encode a polypeptide comprising the PN2-3 to fungal or protozoan cells.

[0076] In one embodiment of the invention, the viral vector preferentially infects the target cells. In another embodiment of the invention, the viral vector preferentially replicates in the target cells, particularly rapidly dividing cells, such as cancer cells. Expression of PN2-3 in cells using a viral vector may also be modulated using a cell-type or cell-cycle-specific promoter, as well as other methods of regulating gene expression, described or referenced herein and known in the art.

[0077] The methods and compositions of the invention described herein may be combined with any other treatment or therapy for treating cancer, for example, chemotherapy, radiation therapy, surgery, and combinations thereof.

[0078] Because microtubules are ubiquitous in eukaryotic cells, the various embodiments of the invention described herein, and other embodiments that will become apparent to the skilled artisan based on the instant disclosure, can be applied to wide variety of different eukaryotic cells. The eukaryotic cells may be animal cells (including both protozoan species and multicellular animals), plant cells (including both algae and multicellular plants), or fungi. Such cells may exist in a multicellular organism, as endogenous cells, commensals, or parasites, or in culture.

[0079] In one embodiment of the invention, the target cells are tumor or neoplastic cells. The tumor cells may be metastatic. The tumor cells may be present in an animal or a plant. In a preferred embodiment, the animal is a non-human primate, canine, feline, bovine, ovine, porcine, or rodent. In another preferred embodiment, the animal is a human. The plant may be an economically important dicot or monocot, including but not limited to, fruit producing plants, grain crops, tubers, plants used to produce economically important medicines, herbs, recreation drugs, and variants and hybrids thereof. In particular embodiments of the invention, the plant is tobacco, corn, wheat, rice, barley, tomato, potato, or hybrids thereof.

[0080] In another embodiment of the invention, the target cells are mycotic (*i.e.*, fungal) cells or protozoan cells, including but not limited to fungal or protozoan cells that infect immunocompromised and/or immunocompetent plants or animals. Examples of fungal cells that can be killed in the practice of the instant invention include but are not limited to *Candida*, *Coccidioides*, *Paracoccidioides*, *Cryptococcus*, *Histoplasma*, *Blastomyces*. *Rhinosporidium*, *Laboa*, *Entomophthora*, *Aspergillis*, *Rhodotolura*, *Trichosporon*, *Monosporium*, *Acremonium*, *Leptosphaeria*, *Geotrichum*, *Saccharomyces*, *Sporothrix*, *Exophilia*, *Madurella*, *Fusarium*, *Wangiella*, *Fonsecaea*, *Phialophora*, *Cladosporium*, *Tinea*, *Allescheria*, *Pseudoallescheria*, *Petriellidium*, *Epidemophyton*, *Pityrosporum*, *Mucor*, *Rhizopus*, *Absidia*, and *Cunninghamella* species. Additional examples include mushrooms and other edible fungi. Examples of protozoan cells that can be killed in the practice of the instant invention include but are not limited to *Pneumocystis*, *Giardia*, *Cryptosporidium*, *Entameoba*, *Endolimax*, *Iodameoba*, *Dientameoba*, *Toxoplasma*, *Malaria*, *Trichomonas*, *Balantidium*, and *Leishmania* species.

[0081] In another embodiment of the invention, the invention provides a method of screening drugs that modulate microtubule assembly or CPAP activity. In one embodiment, drug screening is performed by incubating a drug or test compound, along with a PN2-3 polypeptide, in an assay such as those described herein. In a particular embodiment of the invention, drugs are added to the assay in the presence or absence of PN2-3 polypeptides, and microtubule assembly is determined. In this manner, the practitioner can determine whether a drug inhibits or stimulates microtubule formation, and whether such inhibitory or stimulatory activity is dependent on the presence of a PN2-3 polypeptide. For example, if the drug modulates microtubule assembly only in the presence of a PN2-3 polypeptide, it is likely that the drug affects PN2-3 activity rather than modulating microtubule assembly directly. In this manner, the activity and mechanism of drugs that modulate microtubule formation may be determined.

[0082] The invention also provides antibodies that recognize a PN2-3 polypeptide. Methods of producing antibodies to polypeptides are well known in the art. Such methods have been described, for example, by Harlow and Lane (1988). Antibodies of the instant invention are useful, for example, for PN2-3 polypeptides within a cell to determine whether such polypeptides are associated with centrosomes. Antibodies specific for PN2-3 polypeptides can also be used to stain tissues of animals subject to the various embodiments of the invention to determine whether PN2-3 polypeptides are accumulating specifically or preferentially in the target cell type, thereby monitoring the *in vivo* localization of PN2-3 polypeptides.

[0083] The instant invention also includes pharmaceutical compositions, which contain, as an ingredient, one or more of the polypeptides and/or polynucleotides described herein. In one embodiment of the invention, the pharmaceutical composition comprises PN2-3 polypeptides. In another embodiment of the invention, the pharmaceutical composition comprises a polynucleotide encoding such a polypeptide. In preparing the pharmaceutical compositions of the invention, the polypeptides or polynucleotides are usually mixed with an excipient, diluted by an excipient, and/or enclosed within a carrier which can be in the form of a capsule, sachet, paper or other container. Any carriers or vehicles can be used that facilitate the administration of pharmacological agents, including the polynucleotides and polypeptides of the invention, to a target population of cells. Pharmaceutical compositions may also comprise appropriate immunosuppressants or immunostimulants.

[0084] When the pharmaceutically acceptable excipient serves as a diluent, it can be a solid, semi-solid, or liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders. Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, sterile water, syrup, and methyl cellulose. Other suitable formulations for use in the present invention can be found in *Remington's Pharmaceutical Sciences*. In the case of the instant invention, sterile injectable solutions, for intratumoral or intravenous delivery, are the preferred pharmaceutical embodiments of the invention. Such pharmaceutical compositions may be packaged into convenient kits providing the necessary materials, instructions, and equipment. The pharmaceutical compositions can be administered in a single dose or in multiple doses through routes of inoculation and methods of delivery that are known in art.

[0085] The invention further provides kits that comprise at least one item selected from the group consisting of the following, particularly items 1-4:

1. a PN2-3 polypeptide, a polynucleotide encoding a PN2-3 polypeptide, and/or a composition comprising the polypeptide or polynucleotide;
2. an antibody recognizing the PN2-3 sequence;
3. a chemotherapeutic agent;
4. a means for delivering polypeptides or polynucleotides into a cell; and
5. an instruction of use.

[0086] While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

EXAMPLES

[0087] In the examples below, the following abbreviations have the following meanings. Abbreviations not defined have their generally accepted meanings:

μg	microgram
μl	microliter
μM	micromolar
2-ME	2-mercaptoethanol (also called β-ME)
ATCC	American Type Culture Collection

ATP	adenosine 5'-triphosphate
CDNA	complimentary DNA
cm^2	square centimeters
CMV	cytomegalovirus
CPAP	centrosomal P4.1-associated protein
CPE	cytopathic effects
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DTT	dithiothrietol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP-C1	a green fluorescent protein plasmid
EGTA	ethyleneglycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid
FACS	fluorescent antibody cell sorting
FBS	fetal bovine serum
FITC	fluorosine isothiocyanate
FPLC	fast-protein liquid chromatography
g	gram (except "x g")
x g	times gravity
G_0/G_1	growth phases between M and S
G_2/M	growth phase-2/mitosis
GST	glutathione-*S*-transferase
GTP	guanosine 5'-triphosphate
HeLa	transformed human cervical carcinoma cells
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
hr	hour
L	liter
M	molar
MEM	Modified Eagle's medium
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mm^2	square millimeters
MOI	multiplicity of infection
n	number (*e.g.*, number of animals or duplicates)
°C	degrees Celsius
PAGE	polyacrylamide gel electrophoresis
PBMC	primary blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PFU	plaque forming units
PIPES	piperazine-*N,N'*-bis(2-ethanesulfonate)
PKR	double-stranded RNA activated protein kinase
rpm	revolutions per minute

RPMI	commonly-used cell culture medium
S	DNA synthesis phase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris-HCl	(hydroxymethyl)aminomethane hydrochloride
U	unit
UV	ultraviolet

Materials and Methods

Plasmid construction, purification of GST-recombinant proteins, and peptide synthesis

[0088] cDNAs encoding different portions of CPAP were fused in-frame to glutathione-*S*-transferase (GST) using the plasmid PGEX-2T (Amersham, Piscataway, NJ) and standard molecular biology methodologies. Overexpression and affinity purification of the resulting GST recombinant proteins was performed as described (Hung *et al.*, 2000).

[0089] cDNAs encoding different portions of CPAP were fused in-frame to the C terminus of GFP in a eukaryotic expression vector, pEGFP-C1 (Clontech, Palo Alto, CA), or to a prokaryotic expression vector, pET24(a)+ (Novagen, Madison, WI) using standard molecular biology methodologies.

[0090] Five synthetic peptides (N, H2, C1, C2, and M), corresponding to amino acid residues 310-329 (peptide N), 327-362 (peptide H2), 363-392 (peptide C1), 393-423 (peptide C2), and 320-390 (peptide M) of CPAP (SEQ ID NO:1) were synthesized and FPLC purified by Advanced ChemTech Inc. (Louisville, KY) or by Genemed Synthesis Inc. (South San Francisco, CA).

In vitro centrosome-dependant microtubule nucleation assay

[0091] Isolation of centrosomes and microtubule nucleation tests were performed as described (Hung *et al.*, 2000). To investigate the effect of different GST-truncated CPAP fusion proteins on microtubule nucleation, purified centrosomes (4 µl per reaction) were pre-incubated with the various truncated GST-CPAP fusion proteins or FPLC purified synthetic peptides for 10 min at 4°C. The reaction mixtures were then incubated in 65 μl RG1 buffer (80 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM GTP) containing 125 μg bovine brain tubulin (Cytoskeleton, Denver, CO) for 4 min at 37°C.

[0092] Microtubules present in the reaction mixtures were fixed in 200 μl 1% glutaraldehyde in RG1 buffer, sedimented onto acid-treated coverslips, and subjected to immunofluoresence analysis using anti-α-tubulin monoclonal antibodies N356 (Amersham, Piscataway, NJ) or FITC-conjugated anti-α-tubulin monoclonal antibodies (DM1A-FITC; Sigma, Saint Louis, MO). Truncated GST-CPAP proteins were detected using an anti-GST polyclonal antibody (Molecular Probes, Cincinnati, OH). The extent of microtubule assembly in each reaction was determined microscopically by comparing the extent of microtubule formation on each coverslip. Decreased microtubule formation in reactions comprising polypeptides derived from CPAP indicated the ability of such polypeptides to inhibit centrosome-dependent microtubule assembly.

Microtubule depolymerization assay

[0093] In this assay, centrosomes are omitted from the above reaction mixtures to determine the effects of various truncated GST-CPAP proteins or synthetic peptides on microtubule polymerization in the absence of centrosomes. Polymerized microtubules were stained using FITC-conjugated anti-α-tubulin monoclonal antibodies, as above. Nocodazole (15 μM) was added to some reactions mixtures as a positive control for microtubule depolymerization. A reduction in the amount of polymerized microtubules in reaction mixtures receiving truncated GST-CPAP proteins or synthetic peptides indicated that such truncated GST-CPAP proteins or synthetic peptides possessed microtubule destabilizing activity.

In vitro microtubule sedimentation assay

[0094] Bovine brain α/β tubulin (15 μM; Cytoskeleton Inc., Denver, CO) was pre-incubated with various amounts of truncated GST-CPAP proteins in a 50 μl reaction mixture comprising RG1 buffer (see Example 2) for 30 min at 37°C. Tubulin polymerization was initiated by adding 50 μl GMRG buffer (RG1 buffer supplemented with 12 mM $MgCl_2$ and 50% glycerol), then incubating the reaction mixtures at 37°C for 1.5 hr.

[0095] The reaction mixtures were then subjected to centrifugation at 300,000 x g for 15 min at 37°C, in a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, CA). Supernatants (S) and pellets (P) were analyzed by SDS-PAGE followed by staining with Coomassie blue.

[0096] In experiments containing taxol, microtubules were pre-polymerized using 25 μM paclitaxel (taxol) for 10 min at 37°C in RG1 buffer supplemented with 4 mM $MgCl_2$, 4 mM ATP, and 4 mM GTP. GST-PN2-2 or GST-PN2-3 recombinant proteins were then added to the pre-polymerized microtubules and incubated for an additional 20 min at 37°C. Following incubation, the reaction mixtures were subjected to centrifugation over a 50 μl glycerol cushion (50% glycerol, 10 μM taxol, and 2 mM GTP in RG1 buffer) at 100,000 x g for 30 min at 37°C, in a TL-100 ultracentrifuge. The samples were then resolved by SDS-PAGE. Increased tubulin in the supernatant fractions (and a corresponding decrease in tubulin in the pellet fractions) was indicative of microtubule-destabilizing activity in the reaction mix.

Cell culture and transfection

[0097] HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum. cDNA clones encoding different portions of CPAP were subcloned, in an appropriate reading frame, into the CMV promoter-driven EGFP-C1 expression vector (Clontech, Palo Alto, CA). Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Cold treatment, immunofluorescence and confocal microscopy

[0098] HeLa cells were grown on coverslips for at least 24 hr. Cold treatment was performed by incubating the cells at 4°C for 1 hr, then replacing the cold medium with medium warmed to 30°C and incubating the cells for 2 min at 30°C. The cells were then fixed with 1% formaldehyde for 30 min at room temperature.

[0099] The fixed cells were incubated in the presence of anti-α-tubulin monoclonal antibodies (N356, Amersham, Piscataway, NJ) and anti-γ-tubulin polyclonal antibodies (Sigma, Saint Louis, MO). Bound anti-α-tubulin monoclonal antibodies (N356) were detected with Alexa 568, a Texas Red-conjugated goat anti-mouse IgG, or Alexa 647, a far-red fluorophore-conjugated goat anti-mouse IgG. Bound anti-γ-tubulin polyclonal antibodies were detected with Alexa 568 (Molecular Probes Inc.). The resulting colorimetric signals were detected using a laser scanning confocal system (MRC 1000, Bio-Rad Laboratories, Hercules, CA).

[00100] The effect of various polypeptides derived from CPAP was determined by evaluating the amounts of mitotic spindle formation in the transfected cells. Decreased mitotic spindle formation indicated the presence of an activity that inhibited microtubule formation or destabilized microtubules.

Flow cytometric analysis

[00101] Transfected HeLa cells were harvested 48 hr following transfection. Both the adherent cells and non-adherent cells present in the culture supernatants were collected and subjected to centrifugation. The resulting cell pellets were resuspended in PBS then fixed in 1% formaldehyde at 4°C for 30 min. Following fixation, the cells were collected by centrifugation, washed, and resuspended in 70% cold ethanol. The resuspended cells were either stored at -20°C overnight or immediately used for FACS analysis.

[00102] FACS analysis was performed by collecting the fixed cells by centrifugation and resuspending the cells in DNA staining solution (100 μg/ml propidium iodide, 0.1% Triton X, and 100 μM EDTA in PBS) for 30 min at room temperature. Cell cycle analysis was performed on a FACS Calibur (Becton-Dickinson, Mountain View, CA) with the signal gated to measure only GFP fluorescence. Ten thousand events were acquired and the percentages of cells in G_0/G_1, S, and G_2/M were determined using the

ModfitLT software (version 2.0, Verity Software House, Inc., Topsham, ME). The results of the FACS analysis are shown in Figure 5. The apparent accumulation of cells with two genome copies (*i.e.*, twice the genetic material of G_0/G_1 cells) is indicative of cell cycle arrest after DNA synthesis but prior to mitosis.

[00103] While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

EXAMPLE 1

Structure-Function Analysis of CPAP

[00104] To determine the activity of various regions of CPAP, polynucleotide sequences encoding truncated forms of the CPAP polypeptide were cloned into a GST-expression vector (PGEX-2T; Amersham, Piscataway, NJ) for expression of GST-truncated CPAP polypeptides. A schematic diagram of the various portions of CPAP that were cloned into the expression vector is shown in Figure 1A.

[00105] The resulting GST-tagged polypeptides were affinity purified. Figure 1B shows a Coomassie-stained polyacrylamide gel on which the affinity-purified polypeptides were analyzed. These GST-truncated CPAP proteins were then tested for their ability to affect microtubule formation in a nucleation assay with purified centrosomes.

[00106] As shown in Figure 1C and Table 1, the formation of microtubule asters (*i.e.*, a radial array of microtubules arising from a centrosome) from the purified centrosomes was inhibited by the GST-CM (corresponding to amino acid residues 132-607 of CPAP) and GST-PN2 (corresponding to amino acid residues 132-422 of CPAP) polypeptides but not by the other GST-truncated CPAP proteins tested in the experiment. Microtubule formation was scored as "low": little or no microtubule formation; "intermediate": moderate microtubule formation; or "high": a high degree of microtubule formation.

[00107] Table 1: Centrosome-dependent microtubule nucleation *in vitro* in the presence of polypeptides comprising portions of CPAP

	Low	Intermediate	High
PBS			X
GST-PN1			X
GST-PN2	X		
GST-A5N			X
GST-CM	X		
GST-A5M2-1			X
GST-A5M2-2			X
GST-A5M2-3			X
GST-A5C			X

[00108] This result suggests that the PN2 region, present only in the GST-PN2 and GST-CM polypeptides, possessed the ability to inhibit centrosome-dependent microtubule nucleation *in vitro*. Quantitative analysis of the inhibitory effect observed in the *in vitro* assay is shown in Figure 1D.

EXAMPLE 2

Structure-Function Analysis of PN2

[00109] To more precisely define the region of PN2 that was responsible for inhibiting centrosome-dependent microtubule nucleation, expression vectors were constructed to express GST-fusion polypeptides comprising one of three sub-regions of PN2. The resulting expression vectors, and the respective polypeptides they encoded, were designated PN2-1, PN2-2, and PN2-3 (Figure 2A). These polypeptides were expressed and purified (Figure 2B), then tested for the ability to inhibit centrosome-dependent microtubule nucleation *in vitro*. As shown in Figure 2C-2E and Table 2, only GST-PN2-3 (corresponding to residues 311-422 of CPAP) possessed the ability to inhibit microtubule assembly, indicating that the portion of PN2 necessary to inhibit centrosome-dependent microtubule nucleation resides in the PN2-3 region.

[00110] Table 2: Centrosome-dependent microtubule nucleation *in vitro* in the presence of polypeptide comprising portions of PN2

	None/low	Intermediate	High
PBS			X
GST-PN2-1			X
GST-PN2-2			X
GST-PN2-3	X		

[00111] The CPAP polypeptide comprises five α-helical coiled-coil motifs, designated I to V, which are punctuated by a helix-disrupting proline residue (Hung *et al.*, 2000; Figure 3A). The PN2-3 of CPAP comprises both an α-helical domain (residues 327-362) and a non-helical structure (residues 363-422). To examine the role of this α-helical structure, the PN2-3 polypeptide was further divided into three sub-regions that comprised either the α-helical motif (PN2-3N), the non-helical motif (PN2-3C), or both (PN2-3M) (Figure 3A).

[00112] GST-fusion polypeptides comprising PN2-3N, PN2-3C, or PN2-3M were expressed and purified (Figure 3B), and tested in the above centrosome-dependent microtubule nucleation assay. As shown in Table 3, GST-PN2-3 showed the greatest level of inhibition of microtubule nucleation from the centrosomes, while GST-PN2-3M and GST-PN2-3C exhibited less inhibition. Accordingly, the polypeptides inhibited microtubule nucleation from the centrosomes with the following relative level of efficacy: GST-PN2-3 > GST-PN2-3M > GST-PN2-3C. No inhibition was observed in the case of GST-PN2-3N, which comprises only the α-helical motif (Figure 3C and Table 3). Together, these results suggested that both the α-helical and the non-helical structures in PN2-3 are required for full inhibition of microtubule nucleation from the centrosomes.

[00113] Table 3: Centrosome-dependent microtubule nucleation *in vitro* in the presence of polypeptide comprising portions of PN2-3

	None/low	Intermediate	High
PBS			X
GST-PN2-3	X		
GST-PN2-3N			X
GST-PN2-3M	X		
GST-PN2-3C		X	

[00114] To further define the amino acid residues and/or polypeptide secondary structures responsible PN2-3 activity, four residues within the putative amino acid sequence of α-helix domain II (residues 327-362) were mutated, using standard molecular biology techniques, to the known helix-disrupting residue, proline. Specifically, Y341, I346, L348, and L353 (referring to the full-length CPAP polypeptide) were individually mutated to proline, as shown in Figure 3F. Using the GCG CoilScan program (The Genetics Computer Group package), the probability that the resulting mutated polypeptides would form α-helices was calculated and normalized to that of the "wild-type" PN2-3 polypeptide (*i.e.*, a polypeptide corresponding to amino acid residues 311-422 of SEQ ID NO:1). The predicted probabilities were 0.01, 0.06, 0.00, and 0.06 for Y341P, I346P, L348P, and L353P, respectively, indicating that the mutated polypeptides were less likely to form α-helices than the wild-type PN2-3 sequence.

[00115] The mutated PN2-3 polypeptides, along with a PN2-3 polypeptide harboring the "wild-type" CPAP amino acid sequence (*i.e.*, based on SEQ ID NO:1), were expressed using pET vectors (Novagen, Inc., Madison, WI) and used in the centrosome-dependent microtubule nucleation assay. As shown in Table 4, only the wild-type and L353P polypeptide showed the ability to inhibit microtubule assembly. These results demonstrate that amino acid residues at positions 341, 346, and 348 (but not 353) are important for PN2-3 activity, presumably due to their structural role in forming or maintaining α-helical domain II.

[00116] Table 4: Centrosome-dependent microtubule nucleation *in vitro* in the presence of mutated PN2-3 polypeptides

	None/low	Intermediate	High
PBS			X
PN2-3	X		
Y341P			X
I346P			X
L348P			X
L353P	X		

[00117] To assess the inhibitory effect of CPAP-derived polypeptides in the absence of GST amino acid sequences, five peptides were synthesized and tested in the centrosome-dependent microtubule nucleation assay. The peptides included the α-helical motif (peptide H2), the non-helical structure (peptides N, C1, and C2), or both (peptide M). The structural relationship between these synthetic peptides and PN2-3, PN2-3N, PN2-3M, and PN2-3C are shown in Figure 3A.

[00118] As shown in Table 5, peptide M inhibited centrosome-dependent microtubule nucleation in a dose dependent manner, with more inhibition at 40 μM peptide than at 20 μM peptide. No effect was observed using the N, H2, C1, or C2 peptides at 20 μM or 40 μM. Together, these results indicated that both the α-helical and non-helical structures in PN2-3 contribute to the inhibition of microtubule nucleation, and that the presence of GST sequences in the fusion polypeptides was not relevant to the inhibition of microtubule formation.

[00119] Table 5: Centrosome-dependent microtubule nucleation *in vitro* in the presence of synthetic peptides comprising portions of PN2-3

	None/low	Intermediate	High
PBS			X
N (20 μM)			X
N (40 μM)			X
H2 (20 μM)			X
H2 (40 μM)			X
M (20 μM)		X	
M (40 μM)	X		
C1 (20 μM)			X
C1 (40 μM)			X
C2 (20 μM)			X
C2 (40 μM)			X

EXAMPLE 3

Microtubule depolymerization

[00120] A potential mechanism by which the PN2-3 region of CPAP inhibits microtubule formation is through sequestration and disassembly of microtubules, as proposed for OP18/stathmin. To determine whether PN2-3 inhibition functions through a similar mechanism, GST-CM, GST-PN2-1, GST-PN2-2, or GST-PN2-3 were added to microtubule nucleation reactions, as above, except that centrosomes were absent in the reactions (see Example 2). Nocodazole, a known microtubule-depolymerization drug, was used a positive control. Polymerized microtubules were disassembled in the presence of GST-CM, GST-PN2-3, peptide M, or nocodazole, while no such effect was observed in the presence of GST-PN2-1 or GST-PN2-2 (data not shown). These results thus indicate that the CM and PN2-3 polypeptides of CPAP cause microtubules to disassemble in the absence of centrosomes.

[00121] Microtubule disassembly was confirmed using the microtubule sedimentation assay. Following centrifugation of the reaction mixtures, relatively soluble tubulin monomers and small multimers are found in the supernatant, while relatively insoluble polymerized tubulin is found in the pellet. To assay the ability of PN2-3-containing polypeptides to destabilize microtubules, reactions comprising 15 μM tubulin were incubated in the presence of increasing amounts of GST-PN2-3 (1.9, 3.8, 7.5, and 15 μM) or GST-PN2-2 (15, 30, or 40 μM), or in the absence of CPAP-derived polypeptides (control; Figure 4A).

[00122] In control reactions in which PN2-derived polypeptides were not present, most tubulin was found in polymerized form in the pellet (first two lanes of each panel). However, the addition of the GST-PN2-3 to the reaction mixtures increased the amount of soluble tubulin, present in the supernatant, in a dose-dependent manner, indicating that tubulin polymers were destabilized or disassembled by PN2-3. As little as 1.9 μM GST-PN2-3 caused a detectable increase in the amount of soluble tubulin, while 3.8 μM resulted in the majority of the tubulin being converted to soluble form. Insoluble tubulin was further reduced when the reaction mixtures were incubated with 7.5 μM PN2-3 and was barely detectable with 15 μM (equimolar with respect to tubulin in the mixture) PN2-3.

[00123] In contrast, even 40 μM GST-PN2-2 had little effect on the distribution of tubulin in the supernatant and pellet. These results indicate that the activity of the PN2-3 polypeptide increased the amount of soluble tubulin present in the centrosome-free reactions.

EXAMPLE 4

PN2-3 destabilizes taxol-stabilized microtubules

[00124] A similar result was also observed in taxol-stabilized microtubules (Figure 4B). Taxol stabilizes polymerized microtubules such that soluble tubulin is virtually undetectable in the reaction mixture. The presence of 15 μM GST-PN2-3, but not GST-PN2-2, resulted in most of the tubulin being converted to a soluble form (Figure

4B), indicating that PN2-3, corresponding to amino acid residues 311-422 of CPAP, has the ability to sequester and destabilize microtubules *in vitro*, in the absence or presence of a microtubule stabilizing substance, such as taxol.

EXAMPLE 5

Microtubule destabilization in cultured cells

[00125] To determine whether CPAP destabilizes microtubules in cells, a series of expression vectors was constructed for expressing green fluorescent protein (GFP)-tagged portions of the CPAP polypeptide, or a GFP-tagged full-length polypeptide. HeLa cells were transiently transfected with the GFP-expression vectors, then cold-treated to increase their sensitivity to microtubule destabilization. As summarized in Table 6, the expression of GFP-CPAP-fl (comprising the full-length CPAP polypeptide, residues 1-1338), GFP-CM (comprising CPAP residues 132-607), or GFP-PN2-3 (comprising CPAP residues 311-422), significantly inhibited microtubule reassembly in cold-treated mitotic cells. These polypeptides localized to mitotic centrosomes but were also present in the cytosol (data not shown).

[00126] Table 6: Cell-based assay for CPAP activity

	None/low	Intermediate	High
PBS			X
GFP-CPAP-fl	X		
GFP-CM	X		
GFP-2-3	X		
GFP-A5C			X
GFP-PN2-1			X
GFP-PN2-2			X
GFP-PN1			X
GFP-A5N			X
GFP-A5M2			X
GFP-CPAP-fl*			X

* reduced expression, perhaps due to a lower copy number of the construct being transfected into the cells

[00127] In contrast, no significant inhibition of microtubule reassembly was observed in cells transfected with GFP-A5C (CPAP residues 1070-1338); GFP-PN2-1 (residues 132-190); GFP-PN2-2 (residues 191-310); GFP-PN1 (residues 1-132); GFP-A5N (residues 423-607); or GFP-A5M2 (residues 607-1070), or in cells transfected with expression vectors that expressed lower levels of GFP-CPAP-fl. In the latter cells, GFP-CPAP was detected on the mitotic centrosomes, but only weakly in the cytosol (not shown). Similar results were also obtained using 293 and SiHa cells (not shown). Taken together, these results showed that the PN2-3 region of CPAP inhibited microtubule reassembly from the centrosome in cold-treated cells, providing evidence that CPAP functions as a microtubule destabilizer in cells.

EXAMPLE 6

Cell cycle arrest

[00128] To determine whether overexpression of the PN2-3 polypeptide induces cell cycle arrest, HeLa cells were transiently transfected with the above GFP-PN2-3 expression vector, or a control plasmid encoding only GFP, then analyzed by flow cytometry, as described in Examples 5 and 6. Based on the DNA content, as determined by propidium iodine staining, cells transfected with the GFP-PN2-3 expression vector (but not cells transfected with the control plasmid) showed progressive accumulation in G_2/M (right-most peaks), with a corresponding decrease of cells in G_0/G_1 (left-most peaks)(Figure 5). GFP-PN2-3-transfected cells also exhibited a change in morphology (to round cells) and stopped proliferating (data not shown). These cell cycle and morphological changes were not observed in cells transfected with the expression vector expressing only GFP, indicating that the PN2-3 polypeptide was responsible for the arrest in mitosis at G_2/M and the accompanying morphological changes.